Population-Specific Association between a Polymorphic Variant in *ST18*, Encoding a Pro-Apoptotic Molecule, and Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is a severe autoimmune blistering disease caused by anti-epithelial antibodies, leading to disruption of cell–cell adhesion. Although the disease is exceedingly rare worldwide, it is known to be relatively prevalent in Jewish populations. The low prevalence of the disease represents a significant obstacle to a genome-wide approach to the mapping of susceptibility genes. We reasoned that the study of a genetically homogeneous cohort characterized by a high prevalence of PV may help exposing associated signals while reducing spurious results due to population sub-structure. We performed a genome-wide association study using 300K single-nucleotide polymorphisms (SNPs) in a case–control study of 100 PV patients of Jewish descent and 397 matched control individuals, followed by replication of significantly associated SNPs in three additional cohorts of Jewish, Egyptian, and German origin. In addition to the major histocompatibility complex locus, a genomic segment on 8q11.23 that spans the *ST18* gene was also found to be significantly associated with PV. This association was confirmed in the Jewish and Egyptian replication sets but not in the German sample, suggesting that *ST18*-associated variants may predispose to PV in a population-specific manner. ST18 regulates apoptosis and inflammation, two processes of direct relevance to the pathogenesis of PV. Further supporting the relevance of *ST18* to PV, we found this gene to be overexpressed in the skin of PV patients as compared with healthy individuals.

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Abbreviations: AB, antibody; cDNA, complementary DNA; GWAS, genome-wide association studies; LD, linkage disequilibrium; MHC, major histocompatibility complex; PV, pemphigus vulgaris; SNP, single-nucleotide polymorphism

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INTRODUCTION

Pemphigus refers to a group of autoimmune, mucocutaneous blistering disorders with an established immunological basis (Bystryn and Rudolph, 2005). Pemphigus vulgaris (PV), the most common pemphigus variant, is characterized by flaccid bullae that appear on skin and mucous membranes and easily rupture to form large painful erosions with little or no tendency to heal.

PV is widely believed to result from the deleterious action of autoantibodies (Abs) directed against the desmosomal cadherins, desmoglein 1 and desmoglein 3, resulting in loss of cell–cell adhesion within the epidermis (Stanley and Amagai, 2006), a phenomenon known as acantholysis (Bystryn and Rudolph, 2005). A number of additional pathogenetic mechanisms have been suggested to be operative in PV-IgG-induced acantholysis, such as increased cell apoptosis (Grando *et al.*, 2009), aberrant cell–cell signaling leading to collapse of the cytoskeleton of basal keratinocytes with consequent shrinkage of these cells (Bystryn and Grando, 2006), actin reorganization, and activation of muscarinic receptors uniquely expressed on basal cells (Grando, 2006; Chernyavsky *et al.*, 2008).

Epidemiologically, PV is more common in women (Mimouni *et al.*, 2008) and is between 3-fold and 45-fold

more prevalent among Jews as compared with other populations (Gazit and Loewenthal, 2004). Apart from ethnic propensity, a number of additional facts argue in favor of a genetic contribution to the pathogenesis of the disease, including familial occurrence and the presence of circulating PV-IgG Abs in healthy first-degree relatives of PV patients (Gazit and Loewenthal, 2004).

A number of candidate gene–driven case–control studies conducted in various populations have revealed associations between pemphigus and HLA class I and II regions, non-HLA genes at the same loci, as well as genes encoding relevant autoantigens (Miyagawa *et al.*, 2002; Gazit and Loewenthal, 2004; Gazit *et al.*, 2004; Slomov *et al.*, 2005; Tron *et al.*, 2005; Capon *et al.*, 2006; Brick *et al.*, 2007; Javor *et al.*, 2009; Shams *et al.*, 2009; Tunca *et al.*, 2010).

In contrast with candidate gene–driven studies, hypothesis-free genome-wide association studies (GWAS) often reveal associations with genes encoding proteins of *a priori* unknown relevance to the disease pathogenesis (Carlson *et al.*, 2004). In the present report, we present the results of case–control GWAS in PV.

RESULTS AND DISCUSSION

Genome-wide association study

The low prevalence of PV is a significant obstacle to a whole-genome association approach for the mapping of susceptibility genes in this disorder. We hypothesized that the study of a genetically homogeneous cohort, characterized by a relatively high prevalence of a globally rare trait, such as the Jewish population (Shifman and Darvasi, 2001; Shifman *et al.*, 2003; Gazit and Loewenthal, 2004), may filter out spurious signals while allowing for significant associations to emerge from a relatively low number of participants. Blood samples and skin biopsies were obtained from all participants according to protocols approved by national and local institutional review boards at all participating institutions, in accordance with the Declaration of Helsinki Principles. The diagnosis of PV was confirmed based upon clinical features, suprabasal separation on histology, positive direct and indirect immunofluorescence microscopy, and/or ELISA detection of anti-desmoglein Abs (Zagorodniuk *et al.*, 2005).

We initially genotyped 100 PV Jewish patients (39 men and 61 women, mean age 52 years) and 400 age-, sex-, and population-matched controls (164 men and 236 women, mean age 69 years) for ~300,000 single-nucleotide polymorphisms (SNPs) across the genome. The average call rate was 99.6%. SNPs were filtered according to three criteria: call rate, minor-allele frequency, and deviation from Hardy–Weinberg equilibrium in the controls. Markers that exhibited a call rate lower than 90% or showed a minor-allele frequency <0.01 were removed from the analysis. Deviation from Hardy–Weinberg equilibrium was examined in the control sample, and markers with a *P*-value <0.001 were discarded. A total of 293,635 SNPs passed the above filters. Principal component analysis (Price *et al.*, 2006) ruled out a significant population stratification effect.

We tested for allele association using the PLINK program (Purcell *et al.*, 2007). Figure 1 depicts significant associations found across the genome, demonstrating a high concentration of highly associated SNPs near the major histocompatibility complex (MHC) locus. Out of 82 markers that were identified as significantly associated with PV under a controlled false discovery rate of 0.05 (BH), 69 were found to be located on 6p21.3, within the boundaries of the MHC locus (Supplementary Table S1 online), as demonstrated previously in several studies (Miyagawa *et al.*, 2002; Slomov *et al.*, 2003; Gazit *et al.*, 2004; Brick *et al.*, 2007; Shams *et al.*, 2009; Tunca *et al.*, 2010). The 69 SNPs found on 6p21.3 are distributed within four highly associated peaks

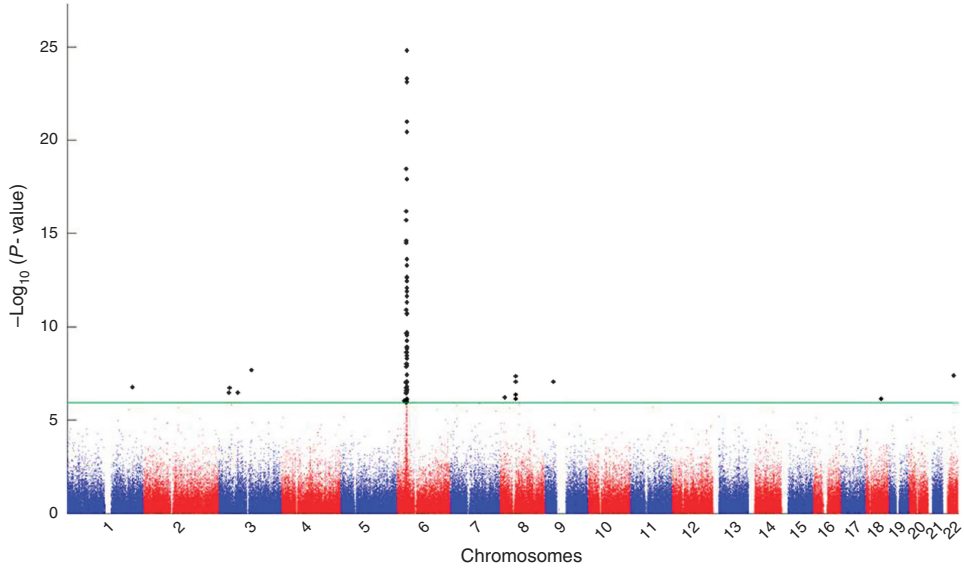

Figure 1. Genome-wide association results. Manhattan plot presenting the negative log-transformed *P*-values from the discovery genome-wide association study plotted against their genomic location. The data above the green line are statistically significant after correction for multiple testing (false discovery rate <0.05).

(depicted in details in Supplementary Figure S1 online) containing a number of genes of interest: *MICB*, *NOTCH4*, *BTNL2*, and the genes encoding HLA-DQA1, HLA-DQB1, and HLA-DRB loci. HLA-DQA1, HLA-DQB1, and HLA-DRBs loci have been described in association with PV in previous studies in both Jewish and non-Jewish populations (Ahmed *et al.*, 1993; Bordenave *et al.*, 2001; Miyagawa *et al.*, 2002; Slomov *et al.*, 2003; Gazit and Loewenthal, 2004; Tron *et al.*, 2005; Brick *et al.*, 2007; Shams *et al.*, 2009). The other three gene loci have not yet been reported in association with PV but harbor variants predisposing to other inflammatory and/or skin disorders (Simmonds *et al.*, 2006; Fernandez-Morera *et al.*, 2008; Okuyama *et al.*, 2008; Barcellos *et al.*, 2009; Cialfi *et al.*, 2010; Clancy *et al.*, 2010; Hsueh *et al.*, 2010; Jin *et al.*, 2011; Juyal *et al.*, 2011).

A number of additional loci outside the MHC region and not previously known to be related to PV also showed significant association with PV. The 10 most significantly non-MHC-associated regions are given in Table 1.

Replication studies

These 10 markers were initially tested for association with PV in an independent set of 59 PV Jewish patients and 285 matched controls (patients and controls selected as above).

Two of the tested SNPs (rs1073208 and rs2304365) were found to be significantly associated in a homozygous state with PV in the Jewish replication set (Table 1). The nearest gene to rs1073208 (50 kb) is *GPD1L*, which has been associated with various forms of inherited arrhythmia (London *et al.*, 2007; Makiyama *et al.*, 2008) and does not seem to be of any directly obvious relevance to the pathogenesis of an autoimmune disorder. On the other hand, rs2304365, as well as two other SNPs found to be significantly associated with PV in the original GWAS (rs4074067 and rs10504140; Table 1), map to the *ST18* gene. *ST18* encodes a transcription factor that regulates inflammation and apoptosis (Yang *et al.*, 2008), two processes of direct relevance to the pathogenesis of PV (Ameglio *et al.*, 1999; Bhol *et al.*, 2001; Narbutt *et al.*, 2008; Javor *et al.*, 2009; Schmidt and Waschke, 2009; Bektas *et al.*, 2010). As noted in Table 1, allele frequency of rs2304365 reached statistical significance in the replication set in a homozygous state only (odds ratio = 6.55, one-sided $P = 0.02$), supporting a recessive inheritance model for the disease risk causative sequence variant. Other recent studies suggest that this pattern of association may be more common than previously thought, especially when the associated allele is population specific (Barrett *et al.*, 2005; Atzmon *et al.*, 2010).

As previously noted, PV is significantly more prevalent in the Jewish population (Gazit and Loewenthal, 2004), raising the possibility that the association with *ST18* is population specific. To test for this hypothesis, we genotyped rs2304365 in two additional replication sets of Egyptian and German origins. The inclusion criteria for patients and controls assessed in these replication studies were identical to those used for the GWAS discovery set. The diagnosis of PV was posed based upon clinical and pathological features, as well as on the basis of positive direct and indirect immunofluorescence microscopy and/or ELISA detection of anti-desmoglein Abs.

We hypothesized that should the ST18 association signal be population specific, it should gradually fade in relation to the genetic distances separating the Jewish people from the two non-Jewish populations (Atzmon *et al.*, 2010; Behar *et al.*, 2010). We genotyped 124 German and 126 Egyptian PV patients together with 275 German and 246 Egyptian control samples for rs2304365. The homozygous A risk allele showed evidence of risk enrichment in the Egyptian sample (odds ratio = 2.25, one-sided P-value = 0.048) but not in the German population (odds ratio = 1.11, one-sided P-value = 0.5672), suggesting that rs2304365 may predispose to PV in a population-specific manner (Supplementary Table S2 online).

The fact that ST18 was associated with PV in the Jewish and Egyptian cohorts, but not in the German population, raises the possibility that this genetic variation originated in early history, possibly reflecting the separation of modern European communities from Middle Eastern populations during the ''Great Migration'' (Cavalli-Sforza *et al.*, 1994).

ST18 expression in the skin of PV patients

Interestingly, the three SNPs on 8q11.23, found in the GWAS to be significantly associated with PV (rs2304365, rs4074067, and rs10504140), are clustered upstream to the coding sequence of all major isoforms of the *ST18* gene (see Supplementary Figure S2 online for more information on *ST18* structure). Linkage disequilibrium (LD) between pairs of SNPs was evaluated using D′, which was calculated based on control genotyping data using the Haploview software (Barrett *et al.*, 2005). Figure 2 shows the LD structure of the relevant region of *ST18*. The two distal SNPs, rs4074067 and rs10504140, whose association with PV could not be replicated in the second Jewish sample set (Table 1), were found to belong to a different LD block than rs2304365, which is part of an LD region that includes the first coding exons of the *ST18* gene, suggesting that this latter SNP may exert an effect on (or be linked to another mutation influencing) ST18 expression in PV.

To assess this possibility, we ascertained the expression of ST18 in a series of non-lesional skin biopsies obtained from PV ($n = 8$) and healthy ($n = 10$) individuals of Jewish origin. Skin biopsies were obtained before treatment initiation. Staining intensity and pattern were scored by three independent observers blinded to the patient diagnosis (Figure 3). ST18 was found to be expressed mostly within the nucleus as previously reported (Jandrig *et al.*, 2004), reflecting its function as a transcription factor (Yang *et al.*, 2008). ST18 was found to be strongly overexpressed in the non-lesional epidermis of PV patients as compared with controls (P-value = 0.00026). In contrast, ST18 staining intensity in the dermis did not differ between the two biopsy sample sets (not shown). Unfortunately, genotypes were not available for most of the patients who contributed a skin biopsy, and thus a clear correlation between genotype and immunohistochemical phenotype could not be established. It should be pointed out, however, that, as it is the case with most similar kind of association data, the SNP found to be associated with PV in our study may not be causally related to the pattern of

Table 1. The 10 non-MHC most significantly associated SNP markers

					GWAS				Replication set								
					Genotype frequencies				Genotype frequencies			Recessive		Dominant		Additive	
	SNP	Genotype	Risk allele	Nearest gene	Controls	Cases	CMT[1] *P*-value	OR	Controls	Cases	Allelic OR	*P*-value[2]	OR	*P*-value[2]	OR	*P*-value[3]	OR
1	rs1345189	AA	G	*ALCAM*	246/397	51/099	0.0001103	2.53	143/276	29/58	1.17	0.162452	1.88	0.457288	1.08	0.237542	1.18
		AG			132/397	33/099			117/276	23/58							
		GG			019/397	15/099			016/276	06/58							
2	rs1316922	CC	T	*LINGO2*	385/395	84/100	0.0004075	6.86	266/277	57/59	0.78	1	-	0.699269	0.85	0.620177	0.79
		CT			010/395	15/100			010/277	02/59							
		TT			000/395	01/100			001/277	00/59							
3	rs4074067	CC	T	*ST18*	299/397	48/100	0.0004099	2.66	189/285	34/54	1.20	0.295166	1.80	0.370873	1.16	0.242071	1.20
		CT			089/397	44/100			087/285	17/54							
		TT			009/397	08/100			009/285	03/54							
4	rs1378394	AA	G	*ARPP-21*	365/396	77/100	0.0008027	3.88	236/255	44/52	2.45	0.169381	-	0.064125	2.26	0.018431	2.40
		AG			031/396	19/100			019/255	07/52							
		GG			000/396	04/100			000/255	01/52							
5	rs1869403	CC	C	*PTPRG*	036/397	24/100	0.00131	2.25	030/265	07/54	1.13	0.440466	1.17	0.343084	1.19	0.281202	1.14
		CT			172/397	51/100			121/265	26/54							
		TT			189/397	25/100			114/265	21/54							
6	rs1073208	CC	C	*GPD1L*	000/377	05/099	0.001313	3.81	000/275	00/54	2.29	0.038600	2.42	-	-	0.019967	2.42
		CT			030/377	17/099			021/275	09/54							
		TT			347/377	77/099			254/275	45/54							
7	rs10504140	AA	A	*ST18*	009/397	08/100	0.001681	2.57	005/273	02/59	1.31	0.361522	1.88	0.205037	1.34	0.138751	1.33
		AG			083/397	39/100			079/273	20/59							
		GG			305/397	53/100			189/273	37/59							
8	rs11991741	AA	G	*VPS37A*	378/397	81/100	0.002182	4.58	258/280	50/54	1.13	0.297621	5.26	0.63356	0.94	0.40678	1.12
		AG			019/397	18/100			021/280	03/54							
		GG			000/397	01/100			001/280	01/54							
9	rs627685	AA	G	*TCF4*	233/397	35/100	0.002502	2.26	146/276	35/58	0.85	0.459008	1.16	0.881217	0.74	0.741297	0.86
		AG			142/397	49/100			105/276	17/58							
		GG			022/397	16/100			025/276	06/58							
10	rs2304365	AA	A	*ST18*	10/397	09/100	0.002502	2.44	003/273	04/59	1.45	0.020840	6.55	0.230441	1.30	0.060589	1.50
		AG			100/397	44/100			087/273	19/59							
		GG			287/397	47/100			183/273	36/59							

Abbreviations: CMT, correction for multiple testing; GWAS, genome-wide association study; MHC, major histocompatibility complex; OR, odds ratio; SNP, single-nucleotide polymorphism.
[1]After CMT.
[2]Fisher's exact test *P*-value for one-sided alternative deduced from GWAS.
[3]Wald test (additive logistic regression) *P*-value for one-sided alternative.

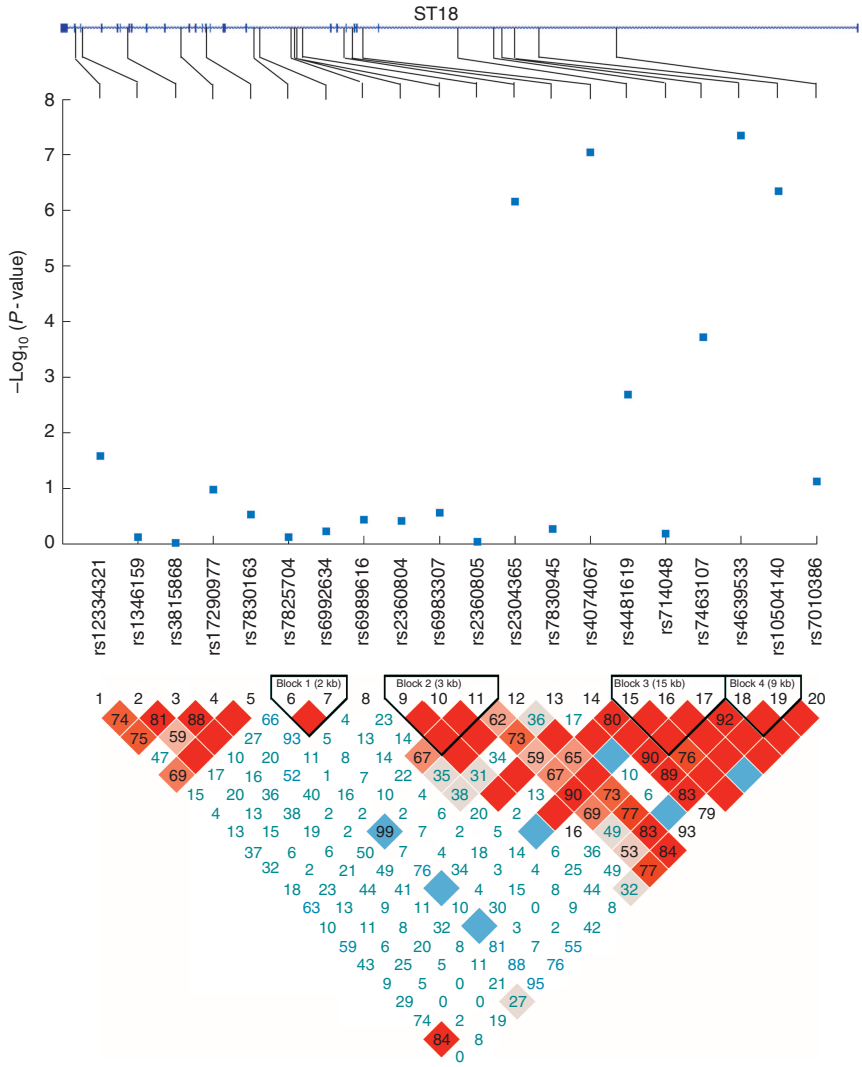

Figure 2. Linkage disequilibrium plot at the 8q11.23 locus. Association results over the 8q11.23 locus are provided as negative log-transformed *P*-values together with the corresponding linkage disequilibrium (LD) values generated using the Haploview software (Barrett *et al.*, 2005). LD levels between the various single-nucleotide polymorphisms across this region are represented by variations in the color of the squares, increasing from white (minimal LD) to bright red (maximal LD). The structure and location of the *ST18* gene is indicated; exons are represented by blue boxes.

ST18 expression, but rather may only point to the existence of truly causative variants located in its vicinity. It is noteworthy that ST18 expression was found to be increased in the epidermis of psoriasis patients too (not shown), suggesting that upregulation of ST18 is associated with inflammatory processes in general and is not restricted to PV.

ST18 expression in peripheral blood lymphocytes did not differ between PV patients as compared with unaffected controls (not shown). However, pathway analysis of a global gene expression study of peripheral leukocyte RNA samples derived from PV patients ($n=9$) and healthy individuals ($n=9$) demonstrated significant enrichment of a network associated with regulation of apoptosis and inflammation (*P*-value = 0.000058), and related to *ST18* (Figure 4, Supplementary Table S3 online, Supplementary Figure S3 online). A significant and central node in the above network is the NF-κB complex (Figure 4), which was shown to regulate tumor

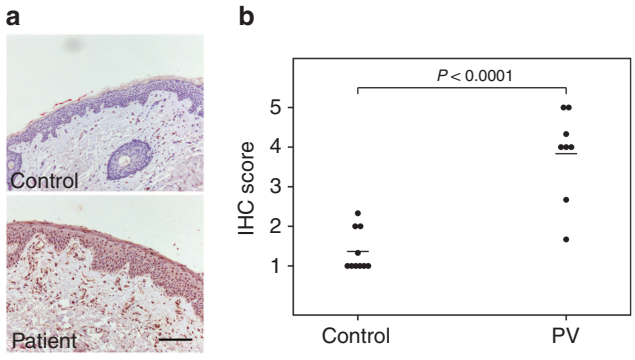

Figure 3. ST18 expression in skin biopsies. (**a**) Tissue sections obtained from pemphigus vulgaris (PV) patients ($n=8$) or from healthy controls ($n=10$) were stained with mouse anti-ST14 and counterstained with hematoxylin (bar = 100 μm). (**b**) Staining intensity was graded from 1 to 5 by three independent observers; mean staining intensity is presented as horizontal line. IHC, immunohistochemistry.

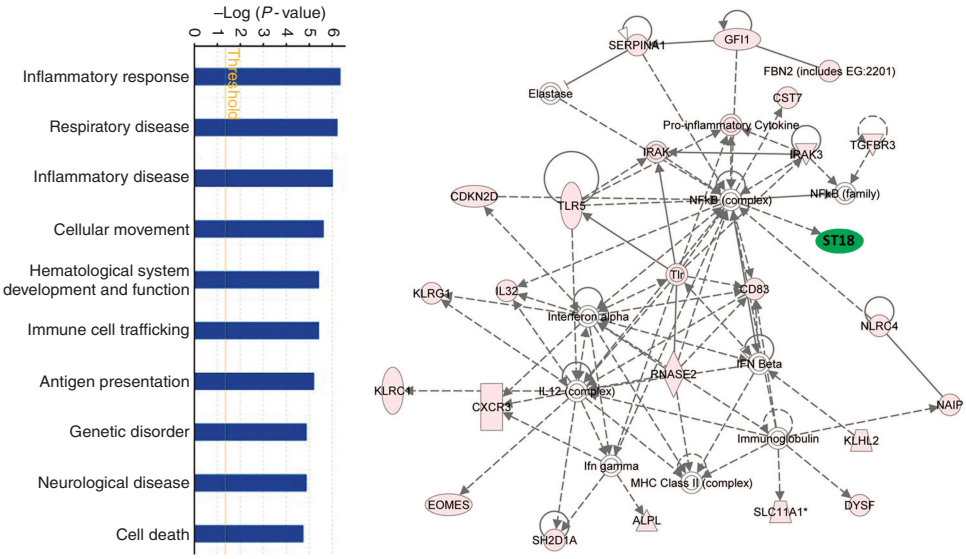

Figure 4. Pathway analysis. The 175 genes that were found to be significantly differentially expressed between cases and controls were used as input for pathway analysis with the ingenuity pathway analysis software. The network that was given the most significant *P*-value and the highest-scored functional pathways is shown. The network was found to be related to ST18 (marked in green).

necrosis factor-α-mediated apoptosis associated with ST18 upregulation (Yang *et al.*, 2008). The fact that tumor necrosis factor-α-mediated apoptosis has been shown to have a major role in PV (Orlov *et al.*, 2006; Bektas *et al.*, 2010) further underscores the relevance of the present genetic and expression findings to PV pathogenesis.

Conclusion

Taken together, the above results point to a significant association between *ST18* and predisposition to PV. Given the importance of apoptosis in PV pathogenesis (Grando *et al.*, 2009) and the role of ST18 in the regulation of this process (Yang *et al.*, 2008), it is possible that ST18 upregulation may specifically influence the susceptibility of epidermal cells to PV-IgG-induced acantholysis.

MATERIALS AND METHODS

Genome-wide association study

The discovery GWAS was conducted using Illumina Human370CNV-Duo BeadChip and HumanHap300 BeadChip (Illumina, San Diego, CA; Salonen *et al.*, 2007). DNA concentration was measured by PicoGreen (Invitrogen, Grand Island, NY), and subsequently 200 ng DNA was hybridized according to the Infinium II assay (Illumina) and scanned with an Illumina beadarray reader (Illumina). The scanned images were imported into BeadStudio 3.1.3.0 (Illumina) for extraction and quality control.

Fisher's exact tests of single-marker association were carried out using PLINK version 1.07 (Purcell *et al.*, 2007) for each SNP. Adjusted *P*-values that correct for the multiple testing performed were calculated using Plink's Benjamini–Hochberg control for false discovery rate. A marker was declared significant under a controlled false discovery rate of 0.05. LD between pairs of SNPs was evaluated using D′, which was calculated based on control genotyping data using HAPLOVIEW, version 4.2 (Barrett *et al.*, 2005).

Follow-up single-SNP genotyping

Single-SNP genotyping was performed with the TaqMan SNP Genotyping Assay kit (Applied Biosystems, Forster, CA) according to the manufacturer's protocol in the 96-well-plate format. We generated one-sided alternative hypotheses for association of these SNPs based on the GWAS results (risk allele identified) and tested them for association in three models: recessive, dominant, and additive. The two former models were tested with Fisher's exact test for one-sided alternative, and the additive model was tested using a Wald test assuming constant increase in log odds for each copy of the risk allele. Both approaches were implemented in R (R Development Core Team, 2010).

Immunohistochemistry

Formaldehyde-fixed 5-μm paraffin-embedded sections were treated with 3% H_2O_2 in methanol for 15 minutes at room temperature, warmed in a microwave oven in citrate buffer for 15 minutes at 90 °C, and stained with a rabbit polyclonal anti-ST18 Ab (LifeSpan Biosciences, Seattle, WA) or preimmune rabbit antiserum for 1 hour at room temperature. After extensive washings in phosphate-buffered saline, the Abs were revealed using the ABC technique (Zymed Laboratories, South San Francisco, CA), and the slides were counterstained with hematoxylin. The intensity of the staining was scored by three independent observers on a scale of 1–5.

RNA extraction and global gene expression

We compared gene expression in a selected group of nine newly diagnosed (untreated) patients and nine sex-, age-, and population-matched control individuals with the Illumina Human-6 v2 expression BeadChip (Illumina, San Diego, CA). RNA was extracted from peripheral blood samples using an RNA extraction kit (Qiagen, Valencia, CA), hybridized to the arrays according to the manufacturer's protocol, and scanned with an Illumina beadarray reader. The scanned images were imported into BeadStudio 3.1.3.0 for extraction and quality control.

The data were logarithmically transformed and normalized with quantile normalization to minimize technical effects. We identified all genes demonstrating >1.75-fold change in expression between each matched sample pair, and considered for further analysis of those genes that demonstrated such changes in more than four sample pairs. Relevant data from the complementary DNA (cDNA) array experiment were validated using real-time RT-PCR across all samples (details below). After validation of the cDNA results, we repeated the pair analysis using a fold cutoff of 1.4. In all, 175 genes were found to be differentially expressed between cases and controls (Supplementary Table S3 online). We then compared the expression profiles between the two groups (cases and controls) using Student's *t*-test, and each of the 175 genes was assigned a *P*-value. This set of data was then ascertained using the ingenuity pathway analysis software (http://www.ingenuity.com), which assigns a *P*-value to each network according to the degree of overrepresentation of input genes as compared with all known genes.

Quantitative reverse transcription PCR

cDNA was synthesized from 500 ng of total RNA using the VERSO kit (Thermo, Waltham, MA), and cDNA PCR amplification was carried out using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St Louis, MO) on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) with gene-specific intron-crossing oligonucleotides (Supplementary Table S4 online).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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